

# Insights into phosphorylation-dependent mechanisms regulating USP1 protein stability during the cell cycle

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**T**ight regulation of the cell cycle and DNA repair machinery is essential for maintaining genome stability. The APC/C<sup>Cdh1</sup> ubiquitin ligase complex is a key regulator of protein stability during the G<sub>1</sub> phase of the cell cycle. APC/C<sup>Cdh1</sup> regulates and promotes the degradation of proteins involved in both cell cycle regulation and DNA repair. In a recent study, we identified a novel APC/C<sup>Cdh1</sup> substrate, the ubiquitin protease USP1. USP1 is a critical regulator of both the Fanconi anemia (FA) and translesion synthesis (TLS) DNA repair pathways. Here, we provide additional mechanistic insights into the regulation of USP1 during the cell cycle. Specifically, we demonstrate that USP1 is phosphorylated in mitosis by cyclin-dependent kinases (Cdks), and that this phosphorylation event may prevent premature degradation of USP1 during normal cell cycle progression. Finally, we provide a unifying hypothesis integrating the role of G<sub>1</sub>-specific proteolysis of USP1 with the regulation of the transcriptional repressors, inhibitor of DNA-binding (ID) proteins.

## Introduction

USP1 is a deubiquitinating enzyme (DUB) with substrates in both the Fanconi anemia (FA)<sup>1</sup> and translesion synthesis (TLS)<sup>2</sup> DNA repair pathways. USP1 regulates the FA DNA repair pathway by deubiquitinating the key effector proteins of this pathway, FANCD2 and FANCI.<sup>3-6</sup> Disruption of USP1 results in DNA cross-linker hypersensitivity, and

USP1-deficient mice possess increased genomic instability and display an FA-like phenotype.<sup>7</sup> To date, there have been no reported cases of patients harboring mutations in the USP1 gene, and it is still not well-understood how deubiquitination of FANCD2 and FANCI promotes repair by the FA pathway. It is possible that (1) deubiquitinating FANCD2 and FANCI removes these proteins from chromatin, making them available for additional repair sites (interestingly, it was shown that the cross-link sensitivity in the absence of USP1 could not be rescued by overexpression of FANCD2 alone.<sup>7</sup> However, attempts to rescue the sensitivity with both FANCI and FANCD2 have yet to be reported) or (2) removal of FANCI and FANCD2 from chromatin may allow the completion of late DNA repair stages.

In contrast to the FA pathway, USP1 actively inhibits the TLS pathway by deubiquitinating PCNA and preventing the recruitment of error-prone TLS polymerases.<sup>8,9</sup> In this setting, USP1 can inhibit mutagenesis and mutagenic repair of plasmids with or without prior exposure to UV damage.<sup>8</sup> Despite the identification of critical USP1 substrates, there is still a lot to learn with respect to how USP1 protects against genomic instability. An obvious place to start is whether USP1's role promoting repair by the FA pathway or inhibiting TLS repair is required to suppress the genomic instability seen in USP1-deficient mice and DT40 cells.<sup>7,10</sup> Furthermore, understanding how USP1 is regulated with respect to different cell cycle stages and following exposure to different DNA-damaging agents will clarify

**Key words:** USP1, phosphorylation, Emi1, Cdh1, APC/C, Cdk, ID

**Abbreviations:** APC/C, anaphase promoting complex/cyclosome; USP1, ubiquitin-specific protease 1; Cdk, cyclin-dependent kinases; UAF1, USP1 associated factor 1; DUBs, deubiquitinating enzymes; FA, fanconi anemia; PCNA, proliferating cell nuclear antigen; SCF complex, Skp1 cullin F-box-containing complex; ID, inhibitor of DNA-binding protein

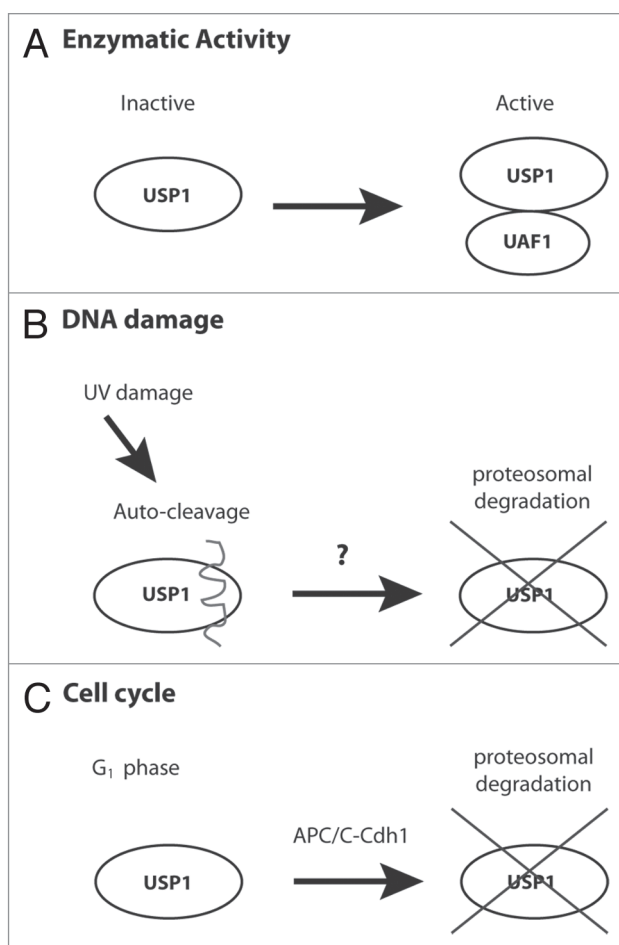
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**Figure 1.** Multiple mechanisms regulate USP1 activity. (A) USP1 requires the association of UAF1 for its full enzymatic activity and protein stability. (B) Upon UV DNA damage, USP1 is auto-cleaved and degraded by the proteasome by an unknown ubiquitin E3 ligase. (C) APC/C<sup>Cdh1</sup> binds to and degrades USP1 during the G<sub>1</sub> phase of the cell cycle.

its role in both of these DNA damage repair pathways.

APC/C<sup>Cdh1</sup> is one of the major ubiquitin ligase complexes involved in regulating the cell cycle.<sup>11</sup> It is typically active during late mitosis and G<sub>1</sub> to promote degradation of positive regulators of mitosis and S phase. In the last few years, several studies have shown APC/C<sup>Cdh1</sup> to have a broader spectrum of substrates.<sup>12</sup> Cdh1-knockout mouse embryonic fibroblasts have substantial chromosomal aberrations and a high degree of genomic instability.<sup>13</sup> We recently expanded the list of substrates that APC/C<sup>Cdh1</sup> targets for degradation by showing that USP1 can interact and be poly-ubiquitinated by APC/C<sup>Cdh1</sup>.<sup>14</sup> More importantly, failure to degrade USP1 (as we previously showed by the expression of a non-degradable mutant) inhibited PCNA mono-ubiquitination during the G<sub>1</sub> UV DNA

damage response and failed to recruit TLS polymerases to sites of DNA damage.<sup>14</sup>

In this review, we will first highlight recent advances made in exploring how the cell cycle regulates USP1 stability and activity. Next, we will include additional data to support the role of Cdk-dependent phosphorylation in the regulation of USP1 protein stability during mitosis. Finally, we will provide new mechanistic insights and propose a working model to explain how and why USP1 needs to be selectively stabilized during mitosis and degraded during the G<sub>1</sub> phase of the cell cycle to ensure genomic stability.

### USP1 is under Tight Control by Multiple Regulatory Pathways

USP1 activity is tightly regulated by several different mechanisms (Fig. 1).

First, the enzymatic activity of USP1 is regulated by binding to a critical catalytic co-factor USP1-associated factor 1 (UAF1), also known as WDR48 or p80.<sup>15</sup> Mutating the WD40 repeats on UAF1 disrupts the USP1-UAF1 interaction and leads to USP1 protein destabilization.<sup>15</sup> UAF1 was also recently shown to regulate the activity of other deubiquitinating complexes containing USP12 and USP46, suggesting a broader role for UAF1 and possibly other WD40 proteins in the regulation of deubiquitinating enzymes.<sup>16</sup> Second, upon UV DNA damage, USP1 is auto-cleaved and degraded by the proteasome.<sup>8</sup> Importantly, under the same conditions, the protein level of UAF1 does not change, and the E3 ubiquitin ligase that targets USP1 for degradation upon UV damage still remains elusive.<sup>15</sup> Finally, we recently reported that USP1 is also regulated in a cell cycle-dependent manner.<sup>14</sup> USP1 is degraded during the G<sub>1</sub> phase of the cell cycle by the ubiquitin ligase APC/C<sup>Cdh1</sup> in order to provide a more permissive environment for mono-ubiquitination of PCNA and TLS recruitment for UV gap repair synthesis.<sup>14,17</sup>

### Regulation of USP1 during the Cell Cycle by APC/C<sup>Cdh1</sup>

APC/C is a multi-subunit E3 ligase complex that is regulated through the binding of one of its two activators: Cdc20 and Cdh1.<sup>18</sup> These activators target substrates at specific stages during mitotic exit.<sup>19,20</sup> Specifically, Cdc20 is bound to the APC/C prior to Cdh1, which binds during later mitotic stages and throughout G<sub>1</sub>. Cdh1 is responsible for the degradation of positive regulators of mitosis and S phase and is considered to be a master regulator of the G<sub>1</sub> phase.<sup>12</sup> In other cell cycle phases, Cdh1 is kept inactive by several mechanisms: (1) Emi1 binding, (2) Cdk-dependent phosphorylation, (3) auto-degradation and (4) degradation by SCF complex.<sup>21-27</sup> Perturbation by these mechanisms allows accumulation of Cdh1 substrates and aberrant cell cycle progression.

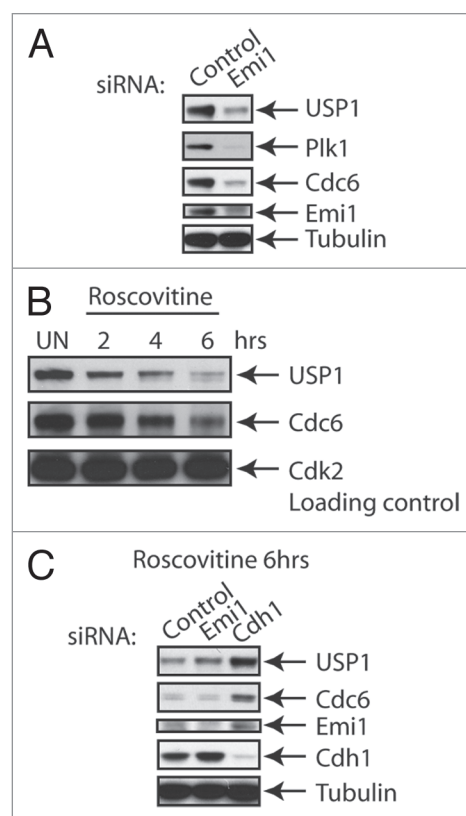
APC/C<sup>Cdh1</sup> also promotes degradation of substrates outside the G<sub>1</sub> phase of the cell cycle. In response to DNA damage, APC/C<sup>Cdh1</sup> can be reactivated in G<sub>2</sub> to target Plk1 for degradation in order

to control the G<sub>2</sub>/M checkpoint.<sup>28</sup> Cdh1 also degrades Rad17, a cell cycle checkpoint protein required for cell cycle arrest that is part of the DNA damage surveillance complex.<sup>29</sup> Additional DNA damage response or repair proteins targeted for degradation by the APC/C<sup>Cdh1</sup> include Claspin, thymidine kinase 1, the ribonucleotide reductase subunit RRM2 and, more recently, USP1.<sup>28,30-32</sup>

In our recent study, we reported that USP1 protein levels oscillate throughout the cell cycle. By synchronizing cells at the G<sub>0</sub>/G<sub>1</sub> boundary with serum deprivation or in prophase by incubation with Nocodazole, we were able to show that USP1 protein levels are downregulated during the G<sub>1</sub> phase of the cell cycle, begin to accumulate rapidly as cells transition into S phase and remain elevated until the late stages of mitosis.<sup>14</sup>

During the G<sub>1</sub> phase of the cell cycle, APC/C<sup>Cdh1</sup> also degrades key cell cycle regulators, such as Cyclin A, Cdc6, Cdt1, Aurora A, Aurora B and Plk1.<sup>33-38</sup> To facilitate the G<sub>1</sub>/S transition, the transcription factor E2F induces the expression of several key cell cycle regulators, including Emi1, a central inhibitor of APC/C.<sup>22,39</sup> Induction of Emi1 expression leads to inactivation of APC/C<sup>Cdh1</sup> through the ability of Emi1 zinc finger domain to interact potently with APC/C<sup>Cdh1</sup> and act as a pseudosubstrate to inhibit its activity and function. Deletion of the zinc domain converts Emi1 into a regular Cdh1 substrates, which can then be degraded by APC/C<sup>Cdh1</sup>.<sup>21,23</sup> Thus, inactivation of APC/C<sup>Cdh1</sup> due to Emi1 expression leads to cyclin A accumulation and further inhibition of APC/C through the phosphorylation of Cdh1. Phosphorylation of Cdh1 by cyclin A-Cdks prevents Cdh1 from binding to the APC/C machinery, resulting in further inactivation of the ligase complex.<sup>24,40</sup> Conversely, loss of Emi1 allows activation of APC/C<sup>Cdh1</sup> prior to S-phase entry and prevents the accumulation of many E2F targets.<sup>41</sup>

To test whether loss of Emi1 can affect USP1 protein levels, we decided to investigate the effect of silencing Emi1 on USP1 protein stability. Silencing of Emi1 in U2OS cells led to a decrease in USP1 and other Cdh1 substrate protein levels, such as Cdc6 and Plk1 (Fig. 2A). However,

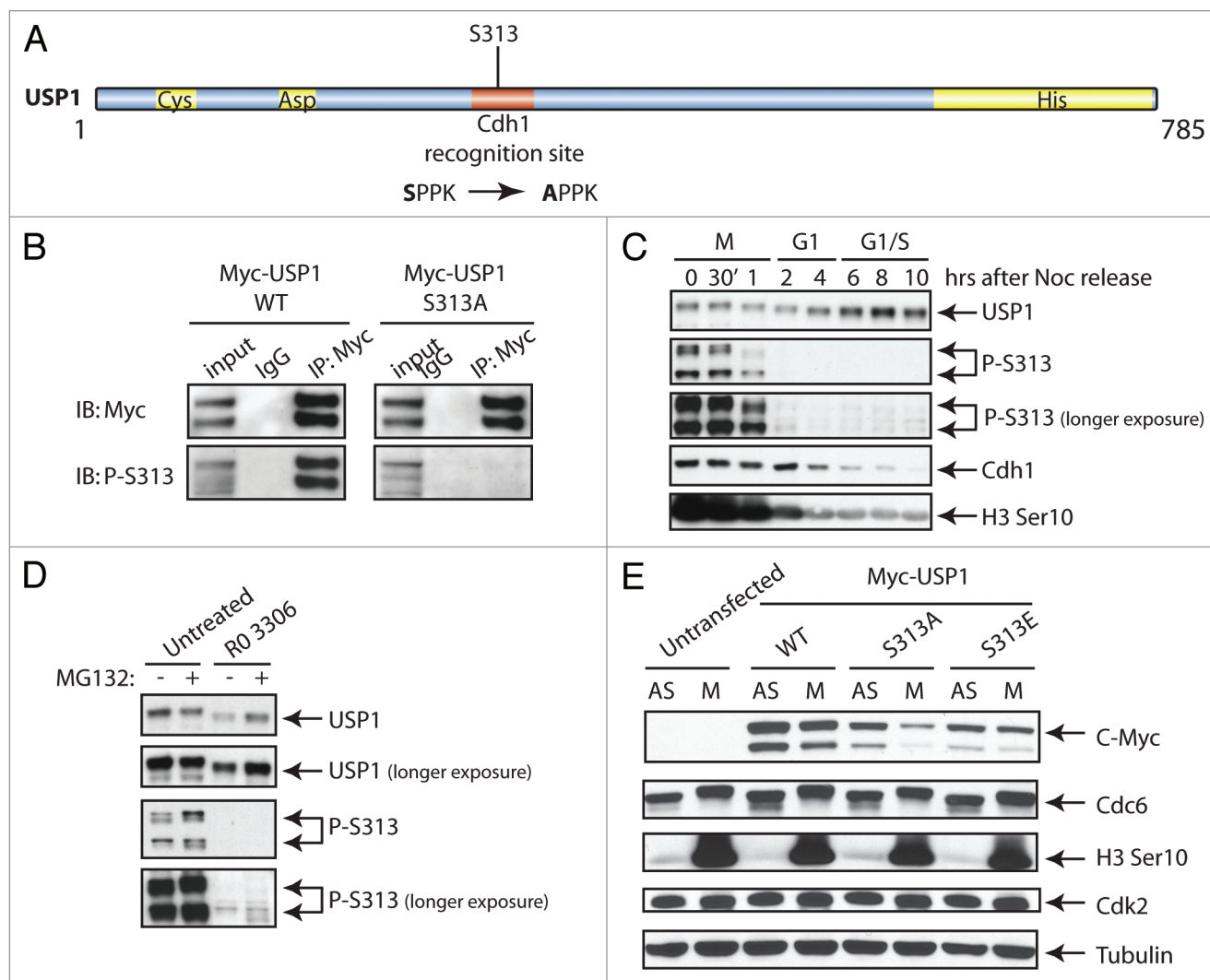


**Figure 2.** Inhibition of Emi1 or Cdks can destabilize USP1. (A) U2OS cells were transfected for 48 h with a control (Ctrl) siRNA oligo (Qiagen AllStar Neg siRNA) or a siRNA oligo directed against Emi1 (5'-CAT GTT CAT TCC GGA CTT AAA-3'). Samples were collected, lysed and analyzed by protein gel blot with the indicated antibodies: Plk1 (Abcam), Cdc6 (Santa Cruz), Emi1 (Invitrogen), Cdk2 (Bethyl), Cdh1 (CalBiochem), Tubulin (Abcam). (B) U2OS cells were left untreated (UN) or treated with Roscovitine (CalBiochem) (5  $\mu$ M) for the indicated time points, analyzed by protein gel blot and probed with the indicated antibodies. (C) U2OS cells were transfected with the indicated siRNAs as in (A) or Cdh1 (AAT GAG AAG TCT CCC AGT CAG) and treated for 6 h with Roscovitine (5  $\mu$ M). Samples were analyzed by protein gel blot and probe with indicated antibodies.

silencing Emi1 and Cdh1 simultaneously led to the rescue of USP1 levels (not shown). This confirmed previous studies, which showed the importance of Emi1 as a Cdh1 inhibitor in order to allow Cdh1 substrate accumulation.<sup>41</sup> Similarly, we also tested the effect of inhibiting Cdks on USP1 protein levels. We treated an asynchronous population of U2OS cells with roscovitine, a purine derivative that can inhibit cyclin-dependent kinase 1 (cdk1), cdk2, cdk5, cdk7 and cdk9.<sup>42</sup> Treatment of cells with roscovitine decreased USP1 protein levels, similar to what has been reported previously for Cdc6 (Fig. 2B).<sup>43</sup> Depletion of Cdh1 in roscovitine-treated cells can rescue the loss of USP1 protein expression (Fig. 2C). Together, this data suggests that both Cdk activity and Emi1 are required to stabilize Cdh1 substrates, including USP1.

### Cdk-Dependent Phosphorylation of USP1 at Serine 313 during Mitosis

Cdk1 is highly active during mitosis and phosphorylates many substrates, including Cdc6.<sup>43</sup> Since we observed a noticeably slower mobility shift in the USP1 protein in U2OS cells arrested in mitosis (not shown), this led us to hypothesize that USP1 might be phosphorylated in M phase, possibly by Cdks. To this end, we searched for putative Cdk phosphorylation sites on USP1 using several publicly available phosphosite databases (Phosida, Phosphosite).<sup>44,45</sup> Among the different phosphorylation sites found on USP1, Serine 313 (S313) was a consensus Cdk site and was identified in all the available phospho-site databases searched. Interestingly, this site is located within the degron region, which we



**Figure 3.** Cdk-dependent phosphorylation of USP1 on S313. (A) Schematic diagram of USP1 catalytic domains, Cdh1 recognition region and putative Cdk phosphorylation site S313 on human USP1. (B) U2OS cells were transfected with Myc-USP1 WT or Myc-USP1 S313A using Fugene6 transfection reagent (Roche Applied Science). Samples were lysed and immunoprecipitated with anti-Myc antibody (Santa Cruz) and protein gel blot was performed using anti-Myc and P-S313 USP1 antibodies (Thermo Scientific). Lysing, immunoprecipitation and washing buffers were done using the low IPB buffer [25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA and 0.5% NP-40 with protease inhibitor cocktail (Roche)]. (C) U2OS cells were synchronized in M phase by incubating cells with Nocodazole (0.1  $\mu$ g/ml) for 12–16 h. After 12–16 h, mitotic cells were shaken off and collected and washed twice with 1x PBS, then plated with fresh media and collected for the indicated time points. Samples were analyzed by protein gel blot and probe with the indicated antibodies: P-S313 USP1 antibody (Thermo Scientific), H3 Ser10 (Millipore) and others described above. (D) U2OS cells synchronized in M phase, as in (C), and were treated (or left untreated) with Cdk1 inhibitor RO-3306 (Calbiochem) (10  $\mu$ M) in the presence or absence of MG132 (10  $\mu$ M). Samples were analyzed by protein gel blot using the indicated antibodies. (E) U2OS cells were transfected as in (C), with Myc-USP1 WT, S313A or S313E, then cells were split, left unsynchronized (AS) or synchronized in mitosis (M). Samples were analyzed by protein gel blot and probe with the indicated antibodies.

previously identified as the Cdh1 recognition site (Fig. 3A).<sup>14</sup> The Cdh1 recognition site within USP1 was previously mapped to amino acids 295–342. We reported that a USP1 deletion mutant, missing amino acids 295–342, is resistant to the G<sub>1</sub> phase APC/C<sup>Cdh1</sup>-mediated degradation.<sup>14</sup> Therefore, we decided to analyze the S313 putative Cdk phosphorylation site and determine whether it is indeed phosphorylated in vivo and can regulate the stability

of USP1. To study the regulation of S313 phosphorylation, we generated an anti-phospho-S313 (P-S313) USP1 antibody. In order to test the antibody specificity, we immunoprecipitated wild-type (WT) Myc-USP1 or Myc-S313A USP1 and immunoblotted with the P-S313 antibody. Figure 3B shows that only the wild-type USP1 was recognized by the phospho-specific antibody, demonstrating that the antibody is specific to the phosphorylated

form of S313. Next, we monitored S313 phosphorylation during mitotic exit by synchronizing U2OS cells in prophase with nocodazole and releasing them into the cell cycle. We observed strong S313 phosphorylation during M phase, and the signal progressively decreased as USP1 was degraded during G<sub>1</sub> (Fig. 3C).

Cdk1 is highly active during mitosis, but this activity is rapidly lost as cyclin B becomes degraded during mitotic exit.



To test whether S313 phosphorylation was Cdk1-dependent, we arrested cells with nocodazole in the absence or presence of the proteasome inhibitor MG132 and the Cdk1 inhibitor RO-3306.<sup>46</sup> Treating prophase-arrested cells with RO-3306 led to the degradation of USP1 as cell exited mitosis (Fig. 3D). This degradation was inhibited with MG132 treatment (as expected), and under these conditions, phosphorylation of USP1 S313 was rapidly lost (Fig. 3D). To begin to understand the role of S313 phosphorylation, we expressed Myc-USP1 WT, S313A or S313E (phospho-mimic mutant) in U2OS cells and tested for their protein stability in mitosis. Myc-USP1 WT and S313E were both stably expressed during mitosis. In contrast, there was reduced protein expression in the S313A when comparing mitotic cells to the asynchronous population, suggesting that the S313A protein may be unstable during M phase (Fig. 3E). Together, these data demonstrate that phosphorylation of USP1 on S313 during M phase is Cdk-dependent and likely important for prolonging USP1 stability during mitosis.

## Discussion

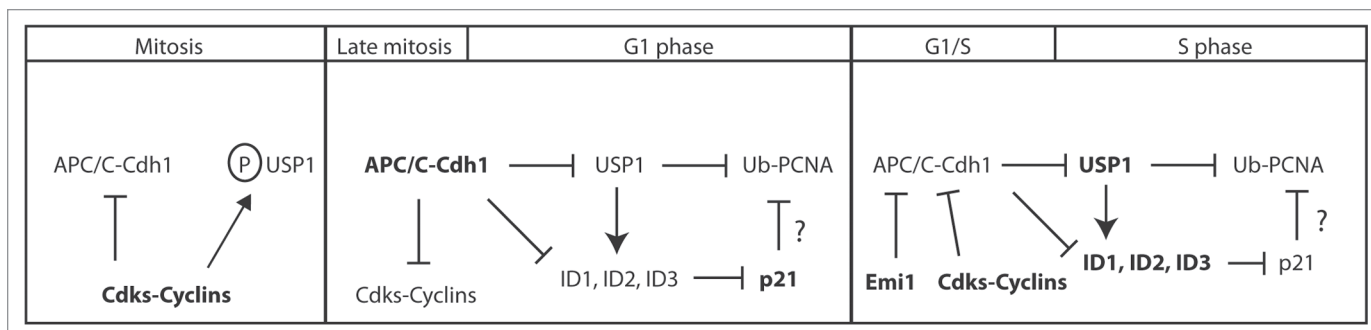
**Possible roles of Cdk-dependent USP1 phosphorylation.** Protein phosphorylation can lead to different regulatory events. Here, we show that USP1 is phosphorylated in M phase by Cdks on S313. We also provide initial evidence that this phosphorylation event may regulate its protein stability. It has been shown that phosphorylation of APC/C substrates can lead to their stabilization; such is the case for Cdc6.<sup>43</sup> Cdc6 is a key regulator for the initiation of DNA replication. Prior to S phase, Cdc6 is kept at low levels by APC/C<sup>Cdh1</sup>-dependent proteolysis.<sup>47</sup> Cdk-dependent phosphorylation of Cdc6 allows for its stabilization by preventing its association to Cdh1.<sup>43</sup> Perhaps phosphorylation of USP1 might also serve as a protective mechanism against APC/C<sup>Cdh1</sup>-mediated degradation. Since the S313 phosphorylation site is located within the region that we previously characterized as the Cdh1 recognition site, we speculate that USP1 phosphorylation on this site prevents its association to Cdh1. More experiments are necessary to prove this point.

Additionally, phosphorylation of USP1 may have other important regulatory functions besides blocking its degradation. Perhaps phosphorylation of USP1 (either on S313 or other uncharacterized Cdk sites) can serve as an interface for interacting with its co-factor UAF1. As we previously reported, APC/C<sup>Cdh1</sup> degrades USP1, but it does not target UAF1.<sup>14</sup> UAF1 levels do not change throughout the cell cycle. UAF1 serves as an enhancer of USP1 activity and stabilizer of USP1 protein levels. Inability to form the UAF1-USP1 complex can lead to USP1 protein destabilization.<sup>15</sup> If phosphorylation of USP1 serves as an interaction site for UAF1, we speculate that once cells exit mitosis, and USP1 becomes dephosphorylated, this might lead to disruption of the USP1-UAF1 complex and subsequent degradation of USP1 by Cdh1 during the G<sub>1</sub> phase of the cell cycle. A recent study showed that phosphorylation of USP37 by Cdk2 leads to its full enzymatic activation in order to allow cyclin A accumulation, APC/C<sup>Cdh1</sup> inhibition and progression into the cell cycle.<sup>48</sup> Perhaps outside the G<sub>1</sub> phase of the cell cycle, phosphorylation is the first step in the activation of USP1, which is then followed by interaction with UAF1 for full activation of its protease activity and stabilization. Therefore, phosphorylation might act as both a protective and activation mechanism for its enzymatic activity. It will be interesting to see whether USP1 activity changes during different cell cycle phases.

**What could be the physiological role of USP1 phosphorylation and G<sub>1</sub>-specific degradation?** It has been reported that FANCD2 and FANCI can form paired mitotic foci in cells undergoing replication stress after treatment with aphidicolin (an inhibitor of replicative polymerase).<sup>49,50</sup> These FANCD2/FANCI mitotic dots colocalize to DNA fragile sites, which are gaps or breaks on chromosomes during mitosis.<sup>49,50</sup> Similar to FANCD2/FANCI, 53BP1, a component of the DNA damage response, has been reported to form larger nuclear foci (53BP1 nuclear bodies) during the G<sub>1</sub> phase of the cell cycle after induced replication stress with low doses of aphidicolin.<sup>51,52</sup> It is presumed that replication stress or incomplete DNA synthesis during S-phase forms FANCD2/FANCI

foci that persist into mitosis and are probably recognized in the next cell cycle phase as DNA lesion, which then results in the formation of 53BP1 nuclear bodies during the G<sub>1</sub> phase. These lesions marked by 53BP1 will be repaired during G<sub>1</sub>, prior to S-phase entry.<sup>51,53</sup> In accordance with this predicted model, the role of USP1 during mitosis may be to deubiquitinate and remove FANCD2/FANCI from their chromatin-bound state. Therefore, USP1 stability (protected against degradation via Cdk-dependent phosphorylation) is critical during mitosis to ensure efficient deubiquitination of FANCD2/FANCI after dealing with replication stress-induced lesions. Then, once the cells enter the G<sub>1</sub> phase, USP1 is subsequently degraded to enable efficient PCNA monoubiquitination and TLS polymerase-dependent repair of lingering DNA lesions via gap repair synthesis. Further studies will be necessary to fully address the role of USP1 phosphorylation during mitosis and how it impacts genomic stability in replicating cells.

**Possible role of USP1-mediated stabilization of ID proteins in TLS.** DUBs have been shown to regulate a wide range of biological processes, and their increasing role in human pathogenesis, such as cancer, underscores the need to target DUBs for therapeutic intervention.<sup>54</sup> A recent study has linked overexpression of USP1 to osteogenic sarcoma and suggested USP1 as a potential therapeutic target.<sup>55</sup> Elegant work by Dixit and colleagues identified three new USP1 substrates called inhibitor of DNA-binding (ID) proteins, ID1, ID2 and ID3. While ID4 is also associated with cancer, its protein stability is likely not regulated by USP1.<sup>55,56</sup> ID proteins antagonize basic-helix-loop-helix (bHLH) transcription factors, such as the Cdk inhibitor p21. They showed that USP1 can interact with ID proteins and prevent their proteasomal degradation.<sup>55</sup> Interestingly, a subset of primary osteosarcomas presented high levels of USP1 and ID proteins concomitantly. Knockdown of USP1 led to the degradation of ID1/2/3, cell cycle arrest and induction of p21. Conversely, overexpression of USP1 led to an increase in ID1/2/3 abundance and cell proliferation.<sup>55</sup> A previous study showed that in neurons,



**Figure 4.** Model showing how USP1-mediated events are regulated during normal cell cycle progression. Schematic diagram of the proposed model of how different cell cycle stages can affect USP1 protein stability, regulation of ID proteins and PCNA-directed DNA repair. Briefly, in M phase, both USP1 and APC/C<sup>Cdh1</sup> are phosphorylated by Cdks, which prevents USP1 from being prematurely targeted for degradation by the APC/C<sup>Cdh1</sup>. In late M and early G<sub>1</sub>, USP1 and APC/C<sup>Cdh1</sup> become dephosphorylated, which leads to the degradation of both USP1 and cyclins. USP1 normally protects ID proteins from ubiquitin-mediated degradation. However, without USP1, ID proteins become subsequently degraded. Loss of ID proteins prevents transcriptional repression of p21, leading to p21 protein accumulation and possible inhibition of TLS activity on PCNA. During the G<sub>1</sub>-S or S-phase entry, levels of cyclins rise to inhibit APC/C<sup>Cdh1</sup>, which lead to the accumulation of USP1 and presumed stabilization of ID proteins.

APC/C<sup>Cdh1</sup> interacts with ID2 and promotes its degradation in cells undergoing quiescence to prevent axonal growth.<sup>57</sup> Therefore, we speculate that during the G<sub>1</sub> phase of the cell cycle, APC/C<sup>Cdh1</sup> targets USP1 for degradation, so it can no longer deubiquitinate the ID proteins, making them susceptible for degradation by APC/C<sup>Cdh1</sup>. This ensures upregulation of p21 and proper G<sub>1</sub> timing to prevent unscheduled DNA synthesis (see model, Fig. 4).

**A unifying hypothesis for USP1 regulation during cell cycle progression and DNA repair.** Here, we present our working model to highlight how different cell cycle phases (namely, M, G<sub>1</sub> and S phase) can regulate both USP1 and ID proteins stability and speculate how these events can ultimately affect PCNA-mediated DNA repair (Fig. 4). (1) In the early mitotic stages, Cdh1 is inhibited by Cdk phosphorylation, which prevents Cdh1 binding to APC/C.<sup>24,25</sup> During mitosis, Cdks can also phosphorylate USP1 on at least S313 (Figs. 3 and 4). We speculate that phosphorylation of USP1 serves as (a) an interaction site for UAF1 and/or (b) a protective mechanism against Cdh1 interaction and subsequent degradation. (2) In the later mitotic stages, Cdh1 is de-phosphorylated, Emi1 gets degraded by SCF<sup>BTRCP</sup> and Cdh1 becomes active and continues the degradation of cyclins.<sup>25,27,58</sup> (3) Once cells enter the G<sub>1</sub> phase of the cell cycle, Cdk activity is very low or absent due to degradation of their cyclin partners by APC/C and therefore

are unable to maintain USP1 phosphorylation (and/or active phosphatase).<sup>33,59</sup> Dephosphorylated USP1 is then susceptible to degradation by APC/C<sup>Cdh1</sup> in the G<sub>1</sub> phase. (4) Low levels of USP1 during the G<sub>1</sub> phase leads to two possible outcomes: first, it allows for a permissive environment for PCNA mono-ubiquitination. Upon DNA damage during the G<sub>1</sub> phase, cells are arrested in G<sub>1</sub> and USP1 degradation by APC/C<sup>Cdh1</sup> is prolonged, allowing PCNA mono-ubiquitination, recruitment of TLS polymerase and DNA repair;<sup>14</sup> second, low levels of USP1 during the G<sub>1</sub> phase of the cell cycle might be unable to inhibit the poly-ubiquitination and degradation of ID proteins by APC/C<sup>Cdh1</sup>. Therefore, degradation of USP1 in G<sub>1</sub> will lead to subsequent degradation of ID proteins. Loss of ID proteins will relieve the transcriptional repression of p21, leading to the accumulation of p21 during G<sub>1</sub>.<sup>55</sup> In the absence of DNA damage, p21 might inhibit PCNA-mediated function during the G<sub>1</sub> phase of the cell cycle by controlling PCNA-interacting TLS polymerases.<sup>60</sup> Several studies have shown that p21 can interact with PCNA and control the loading of TLS polymerases onto PCNA by displacing them.<sup>61-65</sup> (5) At the G<sub>1</sub>/S transition, induction of Emi1 expression inhibits APC/C<sup>Cdh1</sup> activity and leads to the accumulation of APC/C substrates including cyclins, USP1 and, perhaps, ID proteins.<sup>14,22,23,57</sup> Accumulation of cyclins leads to an increase in Cdk-cyclin activity that can further inhibit APC/C<sup>Cdh1</sup>

activity by phosphorylation and allow progression into S phase. (6) In the absence of DNA damage during S phase, USP1 can stabilize ID proteins and transcriptionally repress p21. Since p21 can no longer suppress TLS activity at the replication fork, USP1 becomes more critical in preventing aberrant error-prone TLS polymerase usage in the absence or present of DNA damage.

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